REMARKS

1. Status of the Claims

Claims 1-3, 6-7, 18, 20-24, and 26-31 are pending in the present application. Reconsideration and reexamination of the pending claims is respectfully requested in view of the Remarks below.

2. Rejection Under 35 U.S.C. § 102(e) is Withdrawn

The rejection(s) of claims 1-3 and 6-7 under 35 U.S.C. § 102(e) were withdrawn by the Examiner in view of Applicants' Response mailed November 8, 2004.

Next, the Examiner reminds the Applicants that "if amendment to claim 1 is made in such a way that new matter is removed then rejection to claims 1-3 and 6-7 under 35 U.S.C. § 102(e) would reinstate". Applicants assume that the Examiner means that if the amendments to claim 1 made in the Response filed November 8, 2004 are removed during the course of the present response, then the rejection to claims 1-3 and 6-7 under 35 U.S.C. § 102(e) would be reinstated. Applicants respectfully request clarification of the Examiner's position if Applicants' assumption immediately above is incorrect.

3. New Grounds of Rejection Under 35 U.S.C. § 112

The Examiner rejected claims 1-3 and 6-7 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with 1) the written description requirement and 2) the enablement

requirement. Applicants respectfully traverse the present rejections for the reasons discussed below. First, Applicants will respond to the rejection under the written description requirement and then Applicants will respond to the rejection under the enablement requirement.

a. Rejection of Claims 1-3 and 6-7 Under the Written Description Requirement

Claims 1-3 and 6-7 are rejected under 35 U.S.C. 112, first paragraph, allegedly as failing to comply with the written description requirement.

The Examiner asserts that allegedly "the claims are drawn to an animal that is in need of resistance to endotoxic shock, which has not been disclosed in the specification". Applicants respond that an animal that is in need of resistance to endotoxic shock is disclosed in the specification as follows.

Claims 1 and 6, as originally filed, are related to a method for treating a patient having sepsis in which regulating energy metabolism during a systemic inflammatory response is desired.

Claim 1, as originally filed, is related to "treating a patient having a condition in which regulating energy metabolism during a systemic inflammatory response is desired". The specification defines "systemic inflammatory response syndrome" (SIRS) as "a term which describes the clinical syndrome of sepsis without regard to its cause" (see, e.g., page 2, lines 22-23 of the specification). Furthermore, the

specification states that "the term SIRS is used to denote sepsis, septic shock, sepsis syndrome, and related conditions" (see, e.g., page 3, lines 27-29 of the specification). Still further, the specification refers to "systemic inflammatory response syndrome" conditions as "endotoxic shock", "sepsis", and "septic shock" (see, e.g., page 8, lines 7-9). Therefore, there is support in the specification for "an animal in need to resistance to endotoxic shock" in currently pending claim 1 because originally filed claim 1 is related to treatment during a systemic inflammatory response and because endotoxic shock is defined to be a type of systemic inflammatory response in the specification as set forth above.

By way of further argument, the specification discloses an animal in need of resistance to endotoxic shock at page 23, lines 19-23. Accordingly, the present rejection should be withdrawn because the specification discloses an animal that is in need of resistance to endotoxic shock.

Next, the Examiner asserts that there "is no disclosure when an animal will need resistance to endotoxic shock" (see the bottom of page 3 of the Office Action mailed June 6, 2005 (hereinafter, the "Office Action")). Applicants respectfully disagree because the specification discloses an animal in need of resistance to endotoxic shock and this makes it clear "when" resistance to endotoxic shock was needed (when the animal is in need).

Next, the Examiner asserts that there "is no disclosure ... [of] how long (duration of) resistance to endotoxic shock is needed". Applicants respectfully disagree because one

skilled in the art knows that the duration is as long as the animal is in need of resistance to endotoxic shock. In Example 4 (starting at page 22) of the specification, it is demonstrated that a single administration of a recombinant OB protein was effective in ameliorating endotoxic shock in this Example.

b. Rejection of Claims 1-3 and 6-7 Under the Enablement Requirement

The Examiner asserts on page 6 of the Office Action that "identifying an animal or a patient population in need of conferring resistance to endotoxic shock is highly unpredictable" because "once a bacterial infection has occurred or cytokine levels are high in [an] animal, [it] still does not make an animal in need of resistance to endotoxic shock". This assertion should be withdrawn because it is unsupported by any evidence.

Applicants respectfully submit that one skilled in the art of treating endotoxic shock, which is typically a physician, is capable of determining when an animal is in need of resistance to endotoxic shock using standard methods in the art. There is no inventive skill required. Applicants respectfully submit that the ability to diagnose every single case of endotoxic shock is not a requirement of the claims.

Next, the Examiner asserts at the bottom of page 6 of the Office Action that the "development of an endotoxic shock after an infection or level of cytokine expression would depend on a

number of factors such as severity of infection, duration of infection or level of cytokines in [the] system".

Applicants respectfully submit that the specific factors cited by the Examiner that are allegedly needed to determine if an animal has endotoxic shock are not supported by any evidence of their necessity. Furthermore, one skilled in the art of diagnosing endotoxic shock was capable of accessing the factors cited by the Examiner (if necessary) and other factors to determine whether or not the animal is in need of treatment for endotoxic shock.

Next, the Examiner asserts near the top of page 7 of the Office Action that the working model disclosed in the specification in Example 4 (page 22) of treating an LPS induced endotoxic shock using OB protein allegedly "does not define an animal in need of resistance to endotoxic shock and when one needs this resistance". The Examiner further asserts that one skilled in the art allegedly needs to identify an animal in need of resistance to endotoxic shock, time of administration, and duration of treatment to confer resistance for endotoxic shock and that this would allegedly require huge experimentation and clinical trial to evaluate its validity.

Applicants respectfully submit that the assertions by the Examiner listed above are unsupported by any evidence and, therefore, should be withdrawn. Furthermore, the working model disclosed in Example 4 of the specification is an art recognized model system for studying endotoxic shock. For support please refer, for example, to Shapira et al. (March 1996) Infection and Immunity 64(3):825-828 which states

"Injection of LPS into the bloodstream results in pathophysiological changes that are similar to those seen in sepsis in experimental animals as well as human volunteers" (see page 825, first column, lines 12-15). Please note that sepsis is another term for endotoxic shock. A copy of Shapira is enclosed for the convenience of the Examiner.

Applicants further submit that the Examiner has not established with support that identification of an animal in need of resistance to endotoxic shock and the time and duration of administration would require "huge" experimentation. Also, the Examiner has not provided any evidence that the level of experimentation would be "huge" or that the level of experimentation, if any, required to perform the invention is undue.

> The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. In re Certain Limited-Charge Cell Culture Microcarriers, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), aff 'd. sub nom., Massachusetts Institute of Technology v. A.B. Fortia, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). See also In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404. See MPEP 8th ed. Rev. 3, August 2005 § 2164.01.

Because the Examiner has not established with supportive evidence that undue experimentation is required to perform the claimed invention, the present rejection should be withdrawn.

Referring to the Examiner's assertion that a clinical trial is necessary to evaluate the validity of the enablement of the invention, Applicants respectfully turn to the MPEP quoting the courts which states that clinical trials are not required to demonstrate enablement.

> "[C]onsiderations made by the FDA for approving clinical trials are different from those made by the PTO in determining whether a claim is enabled. See Scott v. Finney, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994) ("Testing for full safety and effectiveness of a prosthetic device is more properly left to the [FDA]."). " See MPEP 8th ed. Rev. 3, August 2005 § 2164.05.

Accordingly, the present rejection based on a requirement for a clinical trial should be withdrawn based on the decision of the Court in Scott v. Finney.

4. Rejection of Claims 18, 20-24 and 27 is Maintained Under 35 U.S.C. § 112, First Paragraph

The Examiner maintained the rejection of claims 18, 20-24, and 27 under 35 U.S.C. § 112, first paragraph, for the reasons of record in the previous Office Actions. The present rejection is respectfully traversed for reasons on record including the Appeal Brief mailed February 12, 2004 (hereinafter the "Appeal Brief"). Please see, for example, pages 7-11 of the Appeal Brief.

Applicants note that the Examiner did not reply to the arguments made in the Appeal Brief in the present Office Action mailed June 6, 2005. Applicants respectfully request that the Examiner respond to the additional arguments made in the Appeal Brief.

5. Rejection of Claims 18, 20-24, 26, and 28-31 is Maintained Under 35 U.S.C. § 103(a)

The Examiner maintained the rejection of claims 18, 20-24, 26, and 28-31 under 35 U.S.C. § 103(a) for reasons on record in the previous Office Actions. The present rejection is respectfully traversed for reasons on record including the Appeal Brief. Please see, for example, pages 12-17 of the Appeal Brief.

Applicants note that the Examiner did not reply to the arguments made in the Appeal Brief in the present Office Action mailed June 6, 2005. Applicants respectfully request that the Examiner respond to the additional arguments made in the Appeal Brief.

CONCLUSION

Claims 1-3, 6-7, 18, 20-24 and 26-31 are currently pending. The Applicants respectfully submit that all pending claims are in condition for allowance and request that the Examiner allow all pending claims.

No new matter is added by way of the present Response.

The Examiner is requested to contact the representative for the Applicants, to discuss any questions or for clarification. If there are any fees associated with this response, the Director is authorized to charge our Deposit Account No. 19-0962.

Respectfully submitted,

Dec. 6, 2005
Date

Michael J. McCarthy, Reg. No 46,910

THE SCRIPPS RESEARCH INSTITUTE
Office of Patent Counsel
10550 North Torrey Pines Road
Mail Drop TPC-8
La Jolla, California 92037
(858) 784-2937

P:\NancyB\WP\2005\PTO\SCR1832P.RESP2FINALOA.wpd

INFECTION AND IMMUNITY, Mar. 1996, p. 825–828
0019-9567/96/$04.00+0

Vol. 64, No. 3

Protection against Endotoxic Shock and Lipopolysaccharide-Induced Local Inflammation by Tetracycline: Correlation with Inhibition of Cytokine Secretion

LIOR SHAPIRA,[1]* W. AUBREY SOSKOLNE,[1] YAEL HOURI,[1] VIVIAN BARAK,[2] AMAL HALABI,[1] AND AYALA STABHOLZ[1]

Hebrew University—Hadassah Faculty of Dental Medicine[1] and Hadassah Medical Center,[2] Jerusalem, Israel

Received 5 July 1995/Returned for modification 31 August 1995/Accepted 7 December 1995

Septic shock results from excessive stimulation of host immune cells, particularly monocytes and macrophages, by lipopolysaccharide (LPS) released from gram-negative bacteria. Macrophage-derived cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β), have been identified as central mediators in the pathogenesis of septic shock and the resultant mortality. Therefore, these cytokines were targets for experimental therapy for septic shock. Because of tetracycline's ability to intervene in cellular mechanisms involved in cytokine secretion, we tested the effect of tetracycline on LPS-induced septic shock and inflammatory lesions in mice. Tetracycline was found to protect mice against LPS-induced lethality and to abolish clinical signs of LPS-induced inflammatory lesions. This protection correlates with tetracycline's ability to reduce LPS-induced TNF-α levels in serum. Furthermore, tetracycline was found to inhibit LPS-induced TNF-α and IL-1β secretion, but not cytokine mRNA accumulation, in human monocytes in vitro. The results presented here suggest that tetracycline is a potent drug for LPS-induced pathology and that its mechanism of action involves blockage of posttranscriptional events of cytokine production.

Septic shock is one of the leading causes of death in hospitalized patients, and mortality rates of up to 50% have been reported (6, 7, 33). Despite all efforts, no regimen today seems to be successful in the treatment of septic shock (3). The shock state results from a systemic infection with gram-negative bacteria (sepsis), but the clinical outcome of the infection leading to septic shock results primarily from the excessive stimulation of the host immune cells, particularly monocytes and macrophages, by lipopolysaccharides (LPS) (6, 35, 36). LPS is a major component of the outer cell wall of gram-negative bacteria, and it is the most potent stimulator of monocyte and macrophage cytokine secretion (36). Injection of LPS into the bloodstream results in pathophysiological changes that are similar to those seen in sepsis in experimental animals (22, 30, 39) as well as human volunteers (20, 25, 26, 28, 45).

Monocyte-derived inflammatory cytokines, particularly tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), have been implicated in the pathogenesis of septic shock (8–10, 14, 18, 22, 24, 27, 30, 31, 37, 39, 44, 50). After injection of LPS, there is a rapid increase in serum TNF-α and IL-1 levels, which peak after 1 to 3 h. This response has similar kinetics in a variety of mammals, including humans (20, 22, 25, 26, 28, 30, 37, 39, 45). In addition, intravenous injection of TNF-α and/or IL-1β was found to result in a septic-shock-like state (2, 13, 32, 49).

Tetracyclines are a well-known family of antibiotics which are active against a wide range of gram-positive and gram-negative bacteria. In addition, anti-inflammatory properties of tetracycline, which are independent of its antibacterial activity, have been described, including the inhibition of protein kinase C and metalloproteinases (15, 19, 52). On the basis of the anti-inflammatory properties of tetracycline, the study presented here was designed to test the hypothesis that tetracycline can be used to prevent LPS-induced pathology and to inhibit cytokine secretion from human monocytes.

* Corresponding author. Mailing address: Department of Periodontics, Hebrew University—Hadassah Faculty of Dental Medicine, P.O. Box 12272, Ein Karem, Jerusalem 91120, Israel. Fax: 972-2-438705. Electronic mail address: shapiral@cc.huji.ac.il.

MATERIALS AND METHODS

Endotoxic shock mouse model. Sabra mice (female, 6 to 7 weeks old, ~35 g) were injected with *Salmonella typhosa* LPS (Sigma, St. Louis, Mo.) intravenously as a model for endotoxic shock. LPS was dissolved in a sterile pyrogen-free saline solution and dispersed by brief sonication. Experimental animals were given 1 ml of a 2-mg/ml tetracycline-HCl solution (58 mg/kg of body weight; Teva, Jerusalem, Israel) by gavage 20 min prior to intravenous LPS injection. Tetracycline administration was repeated 6 and 24 h after the LPS injection but at half of the original dose. Control animals received saline. Mouse mortality was monitored twice daily for 72 h, and in some experiments monitoring was continued once daily for up to 3 weeks.

For the determination of TNF-α levels in serum, mice were challenged with 500 μg of LPS intravenously. Simultaneously, 1 ml of the 2-mg/ml tetracycline-HCl solution was given by gavage to experimental animals, while control animals received saline. The animals were bled from the infraorbital plexus 2 h after LPS challenge, and the levels of TNF-α in the serum were determined by two-site enzyme-linked immunosorbent assay (ELISA) with anti-mouse TNF-α antibodies from Pharmingen (San Diego, Calif.) according to the manufacturer's instructions.

LPS-induced subcutaneous inflammatory lesions. LPS (from *Porphyromonas gingivalis*; 0.1 mg/0.1 ml per animal) was injected subcutaneously on the dorsa of Sabra or BALB/c mice. Simultaneously, the animals were injected intraperitoneally (i.p.) with 0.1 ml of a tetracycline solution (5 to 20 mg/ml) or saline. In some experiments, two groups of control animal were used; one received ampicillin (15 mg/ml; Sigma) i.p. and the other received saline. Lesion size was monitored daily for 3 weeks.

Assessing tetracycline's effect on TNF-α and IL-1β production by LPS-stimulated human monocytes. Fresh human monocytes were isolated from the buffy coats of healthy donors' blood specimens received from the blood bank of Hadassah Medical Center as previously described (42). Briefly, the buffy coats were fractionated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) sedimentation. The mononuclear cell fraction was removed, and the cells were washed three times and resuspended in RPMI 1640 medium supplemented to final concentrations of 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine (all from Biological Industries, Beit-Haemek, Israel), and 2% inactivated human type AB serum (Sigma). The cells were plated in 24-well culture plates at a concentration of 4×10^6 cells per well and incubated for 90 min. Nonadherent cells were removed by aspiration, and the wells were washed three times with phosphate-buffered saline. The adherent cells were then incubated with LPS with or without tetracycline. The medium was collected 18 h after LPS stimulation and kept at −70°C until assayed. TNF-α and IL-1β were assayed in the culture supernatants by two-site ELISA as previously described (42) but

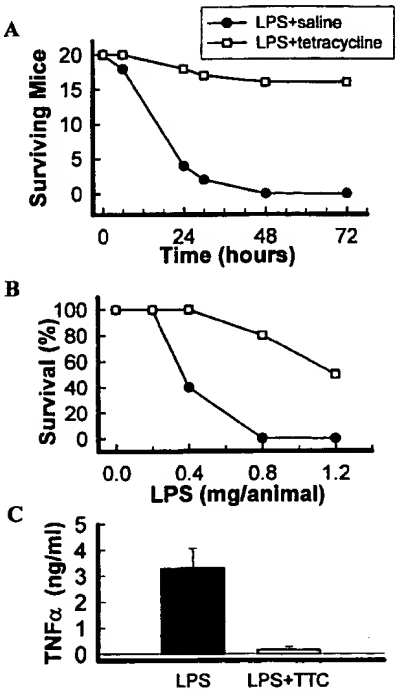

FIG. 1. (A) Survival of mice treated with tetracycline while in LPS-induced septic shock. Mice (n = 20 in each treatment group) were treated as described in Materials and Methods. Mouse mortality was monitored twice daily for at least 72 h. No further mortality was observed between day 3 and day 21 of follow-up. The two treatment groups were significantly different ($P < 0.01$). The same results were observed in three additional experiments, with five mice in each treatment group. (B) Survival of mice treated with tetracycline as described above and challenged with various doses of LPS. Lethality was assessed 48 h later. Each datum point represents 5 to 10 mice. (C) Effect of tetracycline (TTC) treatment on serum TNF-α levels in LPS-injected mice. Mice (n = 3 in each group) were challenged with LPS as described in the text. Animals were bled 2 h after LPS challenge, and the levels of TNF-α in the serum were determined by two-site ELISA. The results are means ± standard errors. The two groups are significantly different (P = 0.016, Student's t test).

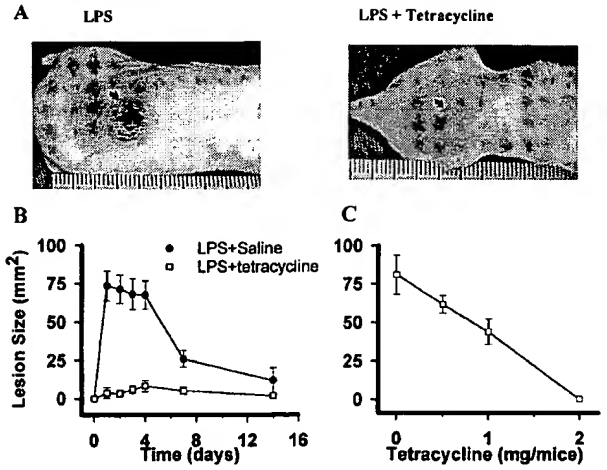

FIG. 2. Effect of tetracycline on LPS-induced lesions in mice. Mice were challenged with LPS as described in the text. Simultaneously, tetracycline was injected i.p. Tetracycline administration was repeated daily for 4 days. Lesion size was monitored daily. (A) Clinical views of the lesion (arrows). The left panel shows a sham-treated mouse with a typical LPS-induced lesion; 75 to 100% of the tetracycline (1.5 mg/day)-treated mice showed no lesion formation. The remaining animals developed minimal-sized lesions like the one illustrated in the right panel. Photographs were taken 72 h after LPS injection. (B) Effect of tetracycline on development of LPS-induced lesions. Mice (n = 5 in each group) were treated with either 1.5 mg of tetracycline per animal per day or saline and were followed up for 14 days. The two groups showed significantly different responses ($P < 0.01$). Similar results were obtained in three more experiments. (C) Dose-dependent inhibition of LPS-induced lesions by tetracycline. Mice (n = 5 in each group) were treated with various doses of tetracycline. Lesion size was measured after 48 h.

with antibodies from R&D Systems (Minneapolis Minn.). TNF-α, IL-1β, and β-actin mRNA were semiquantified by reverse transcription-PCR 2 h after LPS stimulation, as previously described (42, 46), with primers from Clontech (Palo Alto, Calif.) and from Strategene (La Jolla, Calif.) for IL-1β and for TNF-α and β-actin, respectively.

RESULTS

Tetracycline administered by gavage was found to protect mice against a lethal challenge with LPS (Fig. 1A). None of the mice that received 0.8 mg of LPS survived after 48 h, while 80% of the tetracycline-treated mice survived ($P < 0.01$, Z test with the Yates correction). Mice that survived the first 48 h recovered completely and continued to survive for at least another 3 weeks. In addition to significantly reducing mortality under these conditions, tetracycline treatment was found to shift the dose-response curve for LPS-induced lethality to the right (Fig. 1B). Complete protection by tetracycline was observed when the dose of LPS causing 60% mortality was used. Tetracycline administered by gavage to sham-challenged mice did not have a toxic effect.

Oral administration of tetracycline was also found to significantly reduce serum TNF-α levels in LPS-injected mice (Fig. 1C). While the TNF-α level in sera of LPS-challenged control mice was 3.28 ± 0.78 ng/ml (mean ± standard error), the level in tetracycline-treated mice was reduced to 0.14 ± 0.03 ng/ml (n = 3 for each group; P = 0.016, Student's t test).

Examination of the effect of systemic administration of tetracycline on LPS-induced subcutaneous inflammatory lesions in mice also revealed a marked positive effect. Subcutaneous injection of 0.1 mg of LPS into mice induced a visible lesion (60 to 80 mm^2) within 24 h, with tissue necrosis which started to heal spontaneously after 1 week (Fig. 2A and B). Daily administration (i.p.) of tetracycline for the first 4 days following LPS challenge reduced the size of the lesion in a dose-dependent manner (Fig. 2A and C). An optimal effect of tetracycline was seen at a dose of 75 mg/kg of body weight, with total inhibition of lesion formation occurring in 75 to 100% of the treated mice ($P < 0.01$, Z test and one-way analysis of variance). When lesions did occur, their appearance was delayed until the fourth day after LPS challenge, and they were markedly reduced in size (<10 mm^2) and lacked necrosis (Fig. 2A and B). Administration (i.p.) of 100 mg of tetracycline per kg abolished all signs of inflammation but resulted in up to a 20% lethality rate. A unrelated broad-spectrum antibiotic, ampicillin (25 to 100 mg/kg), had no effect on the lesion size (data not shown).

In order to explore some aspects of the mechanism involved, we tested the effect of tetracycline on the secretion of TNF-α and IL-1β by LPS-stimulated human monocytes. Tetracycline was found to inhibit LPS-induced TNF-α and IL-1β secretion into the culture medium in a dose-dependent manner (Fig. 3). Complete inhibition of the secretion of these cytokines was observed with 0.5 mg of tetracycline per ml, and the 50% inhibitory dose was ≈0.1 mg/ml. No cellular toxicity of tetracycline was observed at the tested concentrations. However, when the effect of tetracycline was tested at the mRNA level, no effect of tetracycline on IL-1β mRNA accumulation could be demonstrated in LPS-stimulated monocytes (Fig. 3C). The same results were obtained for TNF-α mRNA (data not shown).

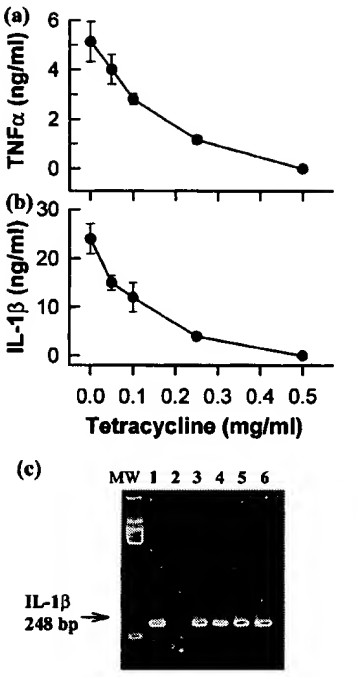

FIG. 3. Dose-dependent inhibition of LPS (1 μg/ml)-induced secretion of TNF-α (a) and IL-1β (b) from human monocytes by tetracycline. Cytokine secretion into the medium was quantified by ELISA. (c) Effect of tetracycline on LPS (1 μg/ml)-induced IL-1β mRNA accumulation in human monocytes. Lane MW, DNA length markers; lane 1, LPS-stimulated monocytes; lane 2, unstimulated monocytes; lanes 3 to 6, monocytes stimulated with LPS and 50, 100, 250, or 500 μg of tetracycline per ml, respectively. mRNA was semiquantified by reverse transcription-PCR.

DISCUSSION

There is little doubt that excess levels of inflammatory mediators lie behind the clinical manifestations and mortality associated with septic shock. Therefore, any intervention that inhibits the release of cytokines or neutralizes their effect is believed to benefit patients experiencing septic shock. The present study showed that treatment with tetracycline modulates the response to LPS and markedly reduces its lethal toxicity in mice. In addition, tetracycline was found to inhibit TNF-α and IL-1β secretion from human monocytes in vitro as well as to reduce serum TNF-α levels in vivo. TNF-α and IL-1β are thought to be central mediators in septic shock, and studies using animal models have shown that targeting TNF-α or IL-1β prevents the clinical manifestation of septic shock (1, 4, 5, 21, 23, 34, 41, 47, 48, 51, 55). However, studies using anti-cytokine strategies in humans were not as successful at saving patients' lives (3). It is reasonable to suggest that more than one mediator is involved in the pathogenesis of septic shock, and targeting a single cytokine might not be as effective as blocking several involved cytokines. The results presented here show that tetracycline inhibits the secretion of both TNF-α and IL-1β from human monocytes. These two mediators were found to be synergistic in the induction of a shock-like state (13, 32, 49). The oral doses of tetracycline that were used in the present study are severalfold lower than the reported 50% lethal dose for tetracycline (16), and no signs of toxicity were detected after oral administration. However, the i.p. injection of high doses of tetracycline (≥2 mg per animal) caused peritoneal injury and death in up to 20% of the animals. It was for this reason that we reverted to the oral administration of tetracycline.

LPS-induced secretion of cytokines from monocytes involves specific intracellular signal transduction events. Several studies show the involvement of protein kinase C, protein tyrosine kinase, and mitogen-activated protein kinase in this process (11, 12, 38, 42, 53, 54). Another step involved in TNF-α secretion is the proteolytic cleavage of membrane-anchored TNF-α and the release of the soluble form of this cytokine (29). This step is dependent on the activity of a specific metalloproteinase. In addition to having antibacterial properties, tetracycline has been shown to inhibit both protein kinase C and metalloproteinase activities (17, 52). The inhibition of these activities was found to be related to the strong chelating ability of tetracycline. It is therefore reasonable to hypothesize that these properties of tetracycline lie behind the mechanism by which cytokine secretion is blocked. The finding that tetracycline inhibits cytokine secretion but not cytokine mRNA accumulation suggests that the mechanism involves the blockage of posttranscriptional events rather than early intracellular events.

Tetracycline has been widely used to treat several localized inflammatory diseases, such as chronic acne and periodontal disease. However, the mechanism of its action is still unclear. It has been suggested that tetracycline is effective at least in part because of its inhibitory effect on tissue collagenase (17). Inhibition of cytokine secretion is another possible pathway by which tetracycline may function in these clinical situations. Indeed, high levels of TNF-α and IL-1β have been associated with tissues affected by periodontal disease, and these cytokines have been implicated as central mediators in the localized destructive process associated with this disease (40, 43).

In conclusion, tetracycline was found to inhibit TNF-α and IL-1β secretion from LPS-stimulated monocytes, to reduce LPS-induced serum TNF-α levels in vivo, to protect mice from experimentally induced septic shock, and to reduce the size of LPS-induced subcutaneous lesions. The data presented here suggest that the use of tetracycline or tetracycline derivatives might be an effective means of therapy in LPS-induced pathologies such as septic shock.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Israel Ministry of Health and the Hebrew University-Hadassah Joint Fund.

REFERENCES

1. **Ashkenazi, A., S. A. Marsters, D. J. Capon, S. M. Chamow, I. S. Figari, D. Pennica, D. V. Goeddel, M. A. Palladino, and D. H. Smith.** 1991. Protection against endotoxic shock by tumor necrosis factor receptor immunoadhesin. Proc. Natl. Acad. Sci. USA **88**:10535–10539.
2. **Bauss, F., W. Dröge, and D. N. Männel.** 1987. Tumor necrosis factor mediates endotoxic effects in mice. Infect. Immun. **55**:1622–1625.
3. **Bernard, G. R.** 1995. Sepsis trials: intersection of investigation, regulation, funding and practice. Am. J. Respir. Crit. Care Med. **152**:4–10.
4. **Beutler, B., I. W. Milsark, and A. C. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229**:869–871.
5. **Bodmer, M., M. A. Fournel, and L. B. Hinshaw.** 1993. Preclinical review of anti-tumor necrosis factor monoclonal antibodies. Crit. Care Med. **21**(Suppl. 10):S441–S446.
6. **Bone, R. C.** 1991. Gram-negative sepsis. Background, clinical features and intervention. Chest **100**:802–808.
7. **Bone, R. C.** 1991. The pathogenesis of sepsis. Ann. Intern. Med. **115**:457–469.
8. **Calandra, T., J. D. Baumgartner, G. E. Grau, M. M. Wu, P. H. Lambert, J. Schellekens, J. Verhoef, and M. P. Glausner.** 1990. Prognostic values of tumor necrosis factor/cachectin, interleukin 1, interferon-alpha and interferon-gamma in the serum of patients with septic shock. Swiss-Ditch J5 immunoglobulin study group. J. Infect. Dis. **161**:982–987.
9. **Creasey, A. A., P. Stevens, J. Kenney, A. C. Allison, K. Warren, R. Catlett, L. Hinshaw, and F. B. J. Taylor.** 1991. Endotoxin and cytokine profile in plasma of baboons challenged with lethal and sublethal *Escherichia coli*. Circ. Shock **33**:84–91.
10. **Debets, J. M., R. Kampmeijer, M. P. van der Linden, W. A. Buurman, and**

C. J. van der Linden. 1989. Plasma tumor necrosis factor and mortality in critically ill septic patients. Crit. Care Med. 17:489–494.
11. Dong, Z., C. A. O'Brian, and I. J. Fidler. 1993. Activation of tumoricidal properties in macrophages by lipopolysaccharide requires protein-tyrosine kinase activity. J. Leukocyte Biol. 53:53–60.
12. Dong, Z., X. Qi, and I. J. Fidler. 1993. Tyrosine phosphorylation of mitogen-activated protein kinases is necessary for activation of murine macrophages by natural and synthetic bacterial products. J. Exp. Med. 177:1071–1077.
13. Everaerdt, B., P. Brouckaert, A. Shaw, and W. Fiers. 1989. Four different interleukin-1 species sensitize to the lethal action of tumor necrosis factor. Biochem. Biophys. Res. Commun. 163:378–385.
14. Fong, Y., K. J. Tracey, L. L. Moldawer, D. G. Hesse, K. B. Manogue, J. S. Kenney, A. T. Lee, G. C. Kuo, A. C. Allison, S. F. Lowry, and A. Cerami. 1989. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1 beta and interleukin 6 appearance during lethal bacteremia. J. Exp. Med. 170:1627–1633.
15. Gabler, W. L., J. Smith, and N. Tsukuda. 1992. Comparison of doxicycline and a chemically modified tetracycline inhibition of leukocyte functions. Res. Commun. Chem. Pathol. Pharmacol. 78:151–160.
16. Goldenthal, E. I. 1971. A compilation of LD50 values in newborn and adult animals. Toxicol. Appl. Pharmacol. 18:185–207.
17. Golub, L. M., N. S. Ramamurthy, T. F. McNamara, R. A. Greenwald, and B. R. Rifkin. 1991. Tetracycline inhibits connective tissue breakdown: new therapeutic implication for an old family of drugs. Crit. Rev. Oral Biol. Med. 2:297–322.
18. Grau, G. E., T. E. Taylor, M. E. Molyneux, J. J. Wirima, P. Vassalli, M. Hommel, and P. H. Lambert. 1989. Tumor necrosis factor and disease severity in children with falciparum malaria. N. Engl. J. Med. 320:1586–1591.
19. Greenwald, R. A., L. M. Golub, B. Lavietes, N. S. Ramamurthy, R. Laskin, and B. Gruber. 1987. Tetracycline inhibits human synovial collagenase in vivo and in vitro. J. Rheumatol. 14:28–32.
20. Hesse, D. G., K. J. Tracey, Y. Fong, K. R. Manogue, M. A. Palladino, A. Cerami, G. T. Shires, and S. F. Lowry. 1988. Cytokine appearance in human endotoxemia and primate bacteremia. Surg. Gynecol. Obstet. 166:147–153.
21. Hinshaw, L. B., P. Tekamp-Olson, A. C. Chang, P. A. Lee, F. B. J. Taylor, C. K. Murray, G. T. Peer, T. E. J. Emerson, R. B. Passey, and G. C. Kuo. 1990. Survival of primates in LD100 septic shock following therapy with antibody to tumor necrosis factor (TNF alpha). Circ. Shock 30:279–292.
22. Klosterhalfen, B., K. Horstmann-Jungemann, P. Vogel, S. Flohe, and F. Offner. 1992. Time course of various inflammatory mediators during recurrent endotoxemia. Biochem. Pharmacol. 43:2103–2109.
23. Lesslauer, W., H. Tabuchi, R. Gentz, M. Brockhaus, E. J. Schlaeger, G. Grau, P. F. Piguet, P. Pointaire, P. Vassalli, and H. Loetscher. 1991. Recombinant soluble tumor necrosis factor receptor proteins protect mice from lipopolysaccharide-induced lethality. Eur. J. Immunol. 21:2883–2886.
24. Marks, J. D., C. B. Marks, J. M. Luce, A. B. Montgomery, J. Turner, C. A. Metz, and J. F. Murray. 1990. Plasma tumor necrosis factor in patients with septic shock. Mortality rate, incidence of adult respiratory distress syndrome, and the effects of methylprednisolone administration. Am. Rev. Respir. Dis. 141:94–97.
25. Martich, G. D., A. J. Boujoukos, and A. F. Suffredini. 1993. Response of man to endotoxin. Immunobiology 187:403–416.
26. Martich, G. D., R. L. Danner, M. Ceska, and A. F. Suffredini. 1991. Detection of interleukin 8 and tumor necrosis factor in normal humans after intravenous endotoxin: the effect of antiinflammatory agents. J. Exp. Med. 173:1021–1024.
27. Mayoral, J. L., C. J. Schweich, and D. L. Dunn. 1990. Decreased tumor necrosis factor production during the initial stages of infection correlates with survival during murine Gram-negative sepsis. Arch. Surg. 125:24–28.
28. Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, and C. A. Dinarello. 1988. Detection of circulating tumor necrosis factor after endotoxin administration. N. Engl. J. Med. 318:1481–1486.
29. Mohler, K. M., P. R. Sleath, J. N. Fitzner, D. P. Cerretti, M. Alderson, S. S. Kerwar, D. S. Torrance, C. Otten-Evans, T. Greenstreet, K. Weerawarna, S. R. Kronheim, M. Petersen, M. Gerhart, C. J. Kozlosky, C. J. March, and R. A. Black. 1994. Protection against a lethal dose of endotoxin by an inhibitor of tumor necrosis factor processing. Nature (London) 370:218–220.
30. Mozes, T., S. Ben-Efraim, C. J. Tak, J. P. Heiligers, P. R. Saxena, and I. L. Bonta. 1991. Serum levels of tumor necrosis factor determine the fatal or non-fatal course of endotoxic shock. Immunol. Lett. 27:157–162.
31. Offner, F., J. Philippe, D. Vogelaers, F. Colardyn, G. Baele, M. Baudrihaye, A. Vermeulen, and G. Leroux-Roels. 1990. Serum tumor necrosis factor levels in patients with infectious disease and septic shock. J. Lab. Clin. Med. 116:100–105.
32. Okusawa, S., J. A. Gelfand, T. Ikejima, R. J. Connolly, and C. A. Dinarello. 1988. Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. J. Clin. Invest. 81:1162–1172.
33. Parrillo, J. E. 1993. Pathogenetic mechanisms of septic shock. N. Engl. J. Med. 328:1471–1477.
34. Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. S. Ohashi, M. Kronke, and T. W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell 73:457–467.
35. Raetz, C. R. H. 1990. Biochemistry of endotoxins. Annu. Rev. Biochem. 59:129–170.
36. Raetz, C. R. H., R. J. Ulevitch, S. D. Wright, C. H. Sibley, A. Ding, and C. F. Nathan. 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. FASEB J. 5:2652–2660.
37. Redl, H., G. Schlag, M. Ceska, J. Davies, and W. A. Burman. 1993. Interleukin 8 release in baboon septicemia is partially dependent on tumor necrosis factor. J. Infect. Dis. 167:1464–1466.
38. Reimann, T., D. Buscher, R. A. Hipskind, S. Krautwald, M. L. Lohmann-Matthes, and M. Baccarini. 1994. Lipopolysaccharide induces activation of the Raf-1/MAP kinase pathway. A putative role for Raf-1 in the induction of IL-1β and TNFα genes. J. Immunol. 153:5740–5749.
39. Remick, D. G., R. M. Strieter, M. K. Eskandari, D. T. Nguyen, M. A. Genord, C. L. Raiford, and S. L. Kunkel. 1990. Role of tumor necrosis factor alpha in lipopolysaccharide-induced pathologic alterations. Am. J. Pathol. 136:49–60.
40. Rossomando, E. F., J. E. Kennedy, and J. Hadjimichael. 1990. Tumor necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. Arch. Oral Biol. 35:431–434.
41. Rothe, J., W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Knotgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann. 1993. Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. Nature (London) 364:798–802.
42. Shapira, L., S. Takashiba, C. Champagne, S. Amar, and T. E. Van Dyke. 1994. Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNFα and IL-1β production by human monocytes. J. Immunol. 153:1818–1824.
43. Stashenko, P., J. J. Jundinski, P. Fujyoshi, J. Rynar, and S. S. Socranski. 1991. Tissue levels of bone resorptive cytokines in periodontal disease. J. Periodontol. 62:504–509.
44. Strieter, R. M., S. L. Kunkel, and R. C. Bone. 1993. Role of tumor necrosis factor-alpha in diseased states and inflammation. Crit. Care Med. 21(Suppl. 10):S447–S463.
45. Suffredini, A. F., R. E. Fromm, M. M. Parker, M. Brenner, J. A. Kovacs, R. A. Wesley, and J. E. Parrillo. 1989. The cardiovascular response of normal humans to the administration of endotoxin. N. Engl. J. Med. 321:280–287.
46. Takashiba, S., T. E. Van Dyke, L. Shapira, and S. Amar. 1995. Lipopolysaccharide-inducible and salicylate-sensitive nuclear factor(s) on human tumor necrosis factor alpha promoter. Infect. Immun. 63:1529–1534.
47. Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami. 1987. Anti-cachectin/tumor necrosis factor antibodies prevent septic shock during lethal bacteremia. Nature (London) 330:662–664.
48. Van Zee, K. J., T. Kohno, E. Fischer, C. S. Rock, L. L. Moldawer, and S. F. Lowry. 1992. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis alpha in vitro and in vivo. Proc. Natl. Acad. Sci. USA 89:4845–4849.
49. Waage, A., and T. Espevik. 1988. Interleukin 1 potentiates the lethal effect of tumor necrosis factor alpha/cachectin in mice. J. Exp. Med. 167:1987–1992.
50. Waage, A., A. Halstensen, and T. Espevik. 1987. Association between tumor necrosis factor in serum and fatal outcome in patients with meningococcal disease. Lancet i:355–357.
51. Walsh, C. J., H. J. Sugerman, P. G. Mullen, P. D. Carey, S. K. Leeper-Woodford, G. J. Jesmok, E. F. Ellis, and A. A. Fowler. 1992. Monoclonal antibody to tumor necrosis factor alpha attenuates cardiopulmonary dysfunction in porcine Gram-negative sepsis. Arch. Surg. 127:138–145.
52. Webster, G. F., S. M. Toso, and L. Hegemann. 1994. Inhibition of a model of in vitro granuloma formation by tetracyclines and ciprofloxacin. Involvement of protein kinase C. Arch. Dermatol. 130:748–752.
53. Weinstein, S. L., M. R. Gold, and A. L. DeFranco. 1991. Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. Proc. Natl. Acad. Sci. USA 88:4148–4152.
54. Weinstein, S. L., J. S. Sanghera, K. Lemke, A. L. DeFranco, and S. L. Pelech. 1992. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. J. Biol. Chem. 267:14955–14962.
55. Windsor, A. C., P. G. Mullen, C. J. Walsh, B. J. Fisher, C. R. Blocher, J. Jesmok, A. A. Fowler, and H. J. Sugerman. 1994. Delayed tumor necrosis factor alpha blockade attenuates pulmonary dysfunction and metabolic acidosis associated with experimental Gram-negative sepsis. Arch. Surg. 129: 80–89.

Editor: J. R. McGhee